Multi-residue analysis of growth promotors in food-producing animals

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CHAPTER 2.1
MULTI-RESIDUE ANALYSIS OF ANABOLIC STEROIDS AND RELATED SUBSTANCES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE ARRAY DETECTION

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Abstract

We describe the development of an HPLC-DAD method for the analysis and identification of 20 anabolic steroids and related substances, that are considered as potential growth promoters, to be used for the analysis of extracts of calf urine samples. First, a suitable column was selected and then a gradient was developed. The compounds are separated on an RP-Select B column using a mobile phase consisting of a mixture of acetonitrile and water. Gradient elution from 43-76% acetonitrile in water using a concave curve was used to achieve a good separation of the compounds with an acceptable analysis time. For the identification of substances, a retention parameter and the UV spectrum were used. The retention parameter was the retention time corrected with a reference mixture. The limits of detection of the HPLC system ranged from 0.5-5 ng injected amount for the androgens, progestagens, stilbenes and resorcylic acid lactones to 5-10 ng injected amount for the oestrogens.

Introduction

Anabolic steroids and some related substances with comparable activities, all of which are here referred to as anabolic steroids, have been used as growth promoters during fattening of cattle for a long time [1,2]. This treatment may result in residues of

the compound in the meat, which could be harmful for the consumer. From sports doping cases and therapeutic uses the anabolic steroids are known carcinogens and prolonged ingestion of larger doses disturb the endocrine balance, leading to a large number of side effects [3]. Although these effects are mainly expected when large doses are ingested, the consumption of meat that contained residues of oestrogenic compounds, has been suggested as the cause of breast enlargement in Italy [4] and precocious puberty in Puerto Rico [5]. Because of these risks, the use of anabolic steroids as growth promoters in cattle was banned in the European Union in 1988 [6]. In the United States and several other countries the natural steroids and zeranol and trenbolone can be used as growth promoters. For the control of the banned substances, samples taken during fattening at the farm or at the slaughterhouse are analysed for the presence of illegal growth promoters. Urine is the sample most often used for the analysis of the anabolic steroids. Analytical methods for human and equine urine [3,7] and for biological samples obtained from food-producing animals [8,9] have been reviewed. Most methods employ solid phase extraction or immuno-affinity chromatography for clean up of the sample and GC-MS for the detection of the steroids [3,7]. However, HPLC has been used for the analysis of anabolic steroids in preparations of illegal growth promoters [10-12] and as a clean up step for biological samples [13,14].

In this chapter, the development of an HPLC-DAD method for the analysis and identification of 20 anabolic steroids is described, which will be used for the analysis of extracts of calf urine samples in the screening for the abuse of illegal growth promoters. The aim of our research was to develop a rapid and cost-effective screening method, which can be used for the analysis of urine samples taken at the farm during fattening. The combination of the information provided by the retention parameter obtained with the HPLC and the UV spectrum recorded with the DAD should be enough to reach an unequivocal identification of the anabolic steroids.

Materials and Methods

Materials

The following chemicals were used for the experiments. As organic modifier in the isocratic HPLC system HPLC grade acetonitrile and methanol (Labscan, Dublin, Ireland) were used. In the gradient HPLC system gradient elution grade acetonitrile (Merck KGaA, Darmstadt, Germany) was used. Water was demineralised in house and was then purified on a Milli-Q system (Millipore NV, Etten-Leur, The Netherlands) or with a Maxima ultrapure water instrument (Elga, obtained from Salm & Kipp BV, Breukelen, The Netherlands). Mobile phases were prepared by mixing demineralised and purified

water with the modifier in the specified proportions (all v/v). Mobile phases were degassed using vacuum and sonication prior to use (vacuum was available through in-house facilities and the Bransonic ultrasonic cleaner model B2210-E-MT was from Bransonic (Bransonic Ultrasonics Corporation, Danbury, CT, USA)).

Steroids and Related Substances

The anabolic steroids used as reference substances were as follows. Methyltesto-sterone (MT) was from Serva (Serva Feinbiochemika GmbH, Heidelberg, Germany). Dienoestrol (DE), hexoestrol (HEX), 17 -ethynyl oestradiol (EE2) and 17ß-oestradiol benzoate (E2benz) were obtained from Sigma (Sigma Chemical Company, St Louis, MO, USA). Medroxyprogesterone (MP) was from Upjohn (Kalamazoo, MI, USA) and androsterone (AD) from Aldrich (Aldrich Chemical Company Inc., Milwaukee, WI, USA). BCR reference standards of zearalenone (Zeara), zeranol (Zer), taleranol (Tal), 19-17 -nortestosterone (NT) and 19-17 ß-nortestosterone (ßNT) were supplied by RIVM (Community Reference Laboratory/Laboratory for Analytical Residue Research, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; further referred to as RIVM). Zeara, ß-trenbolone (Tb), 17ß-oestradiol (E2), stanozolol (Stan), clostebol acetate (ClTac) and clostebol-diol (ClTdiol) standards were supplied by RIVM. Testosterone (T), ßNT, progesterone (P), medrogestone (MED), trans-diethyl-stilbestrol (tDES) and oestrone (E1) were obtained from a local wholesaler.

All stock solutions were prepared in HPLC grade acetonitrile. Calibration standards were prepared in the range of 0.1-250 µg/ml, of which 20 µl were injected, by dilution of the stock solutions with either HPLC grade or gradient grade acetonitrile. The standard solution of tDES contained a 72:28 mixture of tDES and cis-diethylstilbestrol (cDES).

HPLC Equipment

Isocratic HPLC System

The isocratic HPLC system consisted of a Waters model 510 solvent delivery system (Millipore Corporation, Waters Chromatography Division, Milford, MA, USA), with a WISP™ 710B (Waters) automatic sample injection system programmed to make 20 µl injections. The detector was a Kratos Spectroflow 757 absorbance detector (Kratos Analytical Inc, Ramsey, NJ, USA) operated at 241 nm and equipped with a Salm & Kipp recorder type BD 40 04/02 (Salm & Kipp BV).

The columns tested were a Chromsep stainless steel HPLC column (150 x 4.6 mm) packed with Hypersil® ODS material (5 µm) (Chrompack BV, Bergen op Zoom, The Netherlands), a LiChroCART® 250-4 HPLC cartridge Superspher® 60 RP-select B

(Merck), and a LiChroCART® 250-4 HPLC cartridge Superspher® 100 RP-18 (Merck). Guard columns were used to protect the HPLC columns. For the Hypersil column, Chromsep guard columns SS 10x3 mm reversed phase (R3) (Chrompack) were used and for the Superspher columns, LiChroCART® 4-4 HPLC guard columns LiChrospher® 60 RP-select B (5 µm) (Merck).

Gradient HPLC System

The HPLC pump was a System Gold® 126 solvent module (Beckman Instruments Inc., Mijdrecht, The Netherlands) equipped with a System Gold® 168 DAD detector (Beckman). The pump and the detector were controlled with the Gold Nouveau Chromatography Data System® version 1.0 (Beckman, 1996), run on an IBM personal computer 330p100 (Beckman) equipped with a HP deskjet 510 printer (Hewlett Packard, Amsterdam, The Netherlands).

The HPLC column was a LiChroCART® 250-4 HPLC cartridge containing Superspher® 60 RP-select B material, 250x4 mm (Merck) protected by a LiChroCART® 4-4 guard column with LiChrospher® 60 RP-select B material, 4x4 mm (Merck). The injector was a Rheodyne 7725i injector equipped with a 20 µl sample loop (Rheodyne, Cotati, CA, USA).

The flow rate was set at 0.8 ml/min. The gradient was made up from 40% acetonitrile in water (v/v) (solvent A) and acetonitrile (solvent B). For the final system, the solvent module was programmed to deliver the following gradient:
0-5 min: 95% A and 5% B (43% acetonitrile in water (v/v))
5-25 min: gradient from 95% A to 40% A with curve 6
25-30 min: 40% A and 60% B (76% acetonitrile in water (v/v))
30-32 min: linear gradient from 40% A to 95% A (curve 0)
32-45 min: restabilise at 95% A and 5% B (43% acetonitrile in water (v/v)).

The DAD-detector was programmed to collect data for 35 minutes from the start of the run. An autozero scaling was performed at the start of each new run. The scan range was 190-400 nm. Data were collected at a rate of 2 Hz. Readings were performed at 192, 230, 242, 280, or 350 nm (bandwidth 4 nm) depending on the steroid studied. For routine operation, the software can be programmed to collect data at those five wavelengths (multichromatogram mode). Spectra were saved for detected peaks in this mode. Detection wavelengths for the quantitation of steroids were:
192 nm: E2, EE2, E1
230 nm: DE, HEX, cDES, Tal, Zer, sometimes E2 (all steroids can be detected at this wavelength)

242 nm: T, NT, ßNT, MT, tDES, P, MP, ClTac, ClTdiol, Zeara
280 nm: MED
350 nm: Tb

Methods

Isocratic HPLC System

In the first set of experiments the Hypersil ODS column was used with different methanol-water and acetonitrile-water mixtures as mobile phases. The flow was also varied to determine the optimum value. In those experiments ßNT, MT and DES were used as test substances.

In the second set of experiments three HPLC columns (Hypersil ODS, Superspher RP-select B and Superspher RP-18) were compared with a mobile phase consisting of 42% acetonitrile in water at a flow rate of 0.8 ml/min. The mobile phase composition was further optimised for the two Superspher columns to obtain retention times larger than 10 min to separate the steroids from polar matrix components expected in extracts of urine samples and to achieve a good separation of the steroids. In those experiments ßNT, T, MT and DES were used as test substances.

The optimum configuration was found to be a Superspher RP-select B column with a mobile phase consisting of 45% acetonitrile in water at a flow rate of 0.8 ml/min. The linearity of the method was determined with calibration standards in acetonitrile in the range of 0.1-250 µg/ml or 2-5000 ng injected amount (n=6, except for the highest standard n=3). The limits of detection (LODs) were calculated at three times the noise using those calibration curves.

Gradient HPLC System

The experiments were continued with the Beckman System Gold Nouveau HPLC system, equipped with a DAD to provide simultaneous detection of all steroids and which allowed gradient elution. The Beckman software can produce 7 different gradient curves and these were compared with a test mixture containing Tb, E1, MT, Zeara, tDES, HEX, cDES, P and MED at a concentration of about 10 µg/ml (for tDES and cDES the total concentration was 10 µg/ml). The gradient program used in this experiment was 5 min isocratic 40% acetonitrile in water, in 20 min to 67% acetonitrile in water using different gradient curves, 5 min isocratic 67% acetonitrile in water, in 2 min linear gradient back to 40% acetonitrile in water, and finally, 13 min restabilisation. Data collection was stopped at 35 min. Gradient curve 6 was selected as the one best suitable for the separation of the steroids. The percentages of acetonitrile were changed to 43% acetonitrile in

water as initial composition and 76% acetonitrile in water as final composition to optimise the retention times of the steroids. Final HPLC conditions and detector parameters are described in the section 'Gradient HPLC System'.

With this system calibration standards in acetonitrile in the range of 0.25-10 µg/ml or 5-200 ng injected amount were analysed. The peak heights obtained at the specific wavelengths were used to construct calibration curves. The LODs were calculated at three times the noise at the specific wavelength used for those calibration curves.

Calculations

Chromatographic characteristics were calculated by the Gold Nouveau Chromatography Data System. Plate numbers (N) and resolutions (Rs) to the previous peak were calculated according to the DAB method of the software, which uses the equations [15]:

$$N = 5.54 * (RT/w_{0.5})^2$$

$$Rs = 1.18 * (RT_2 - RT_1)/(w_{0.5,1} + w_{0.5,2})$$

where $w_{0.5}$ is the peak width at half maximum for two neighbouring peaks, 1 and 2, respectively.

The selectivity factor ( ) for two neighbouring peaks, 1 and 2, respectively was calculated according to the equation [15]:

$$= k'_2/k'_1$$

Cis-Trans Isomerism of DES

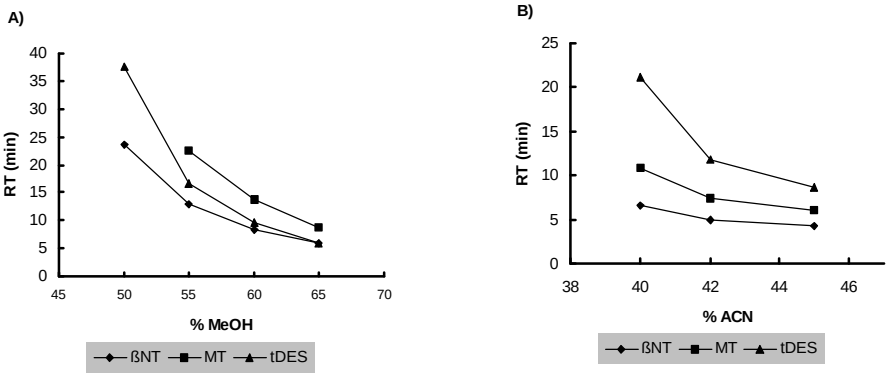
For this experiment a 0.5 mg/ml solution of tDES in acetonitrile was prepared. The solution was analysed by gradient HPLC immediately after preparation and was then placed in a waterbath (Gebr Haake, Berlin, Germany) at 37 °C for 6.5 hours. During this time samples were analysed by HPLC every 45 min to follow the appearance of the new peaks. Thereafter, the solution was kept at room temperature and under the influence of daylight. Samples were analysed on days 1, 3, 6, 9 and 24. The DAD spectra obtained were compared with those found in literature. From the results at t=0 the extinction coefficient of tDES was determined and the extinction coefficient of cDES could be calculated from the data obtained in the isomerisation experiment.

Results and Discussion

Isocratic HPLC System

It was impossible to determine the moment of injection exactly with the isocratic HPLC system, because no connection could be made between injector and recorder. Therefore, the mark button on the detector was pressed at approximately the moment the

Figure 1. The effect of two modifiers on the retention times of three anabolic steroids on a Hypersil ODS column. The percentage modifier was varied from 50-65% methanol in water (A) and from 40-45% acetonitrile in water (B). The flow rate was kept at 1.0 ml/min.



sample was injected. Retention times (RTs) and column dead time were determined from the moment the mark button was pressed. Column dead time was defined as the time required for unretained dissolution solvent to pass through the column. RTs and column dead times could be determined reproducibly (relative standard deviation over different days < 1.5% for column dead times and < 5% for RTs of steroids) with this procedure.

Two modifiers were used to separate an initial mixture of three steroids (ßNT, MT and tDES) on the Hypersil ODS column: Methanol or acetonitrile. The percentage modifier was varied from 50-65% methanol in water and from 40-45% acetonitrile in water. The results are summarised in Figure 1. With a mobile phase of 65% methanol in water ßNT and tDES co-eluted and with a mobile phase of 50% methanol in water the RT of MT became unacceptably long. With 55% and 60% methanol in water the RTs and resolutions were acceptable. When acetonitrile was used as modifier the elution order changed. tDES now eluted after MT. Resolution between the steroids was acceptable with all mobile phases tested, but the RTs were too short with 45% acetonitrile in water, because matrix interferences of extracts of urine extracts are expected to elute within the first 5-7 minutes. With 40% and 42% acetonitrile the RTs of the compounds were acceptable, although the RT of tDES with 40% acetonitrile was quite long. There was not much variation in the plate numbers between the two modifiers, although the values with acetonitrile tended to be better. The flow rate was also varied from 0.5 to 1.5 ml/min with

Table 1. Net retention times (min) and capacity factors of three anabolic steroids and a stilbene on three different HPLC columns with an isocratic mobile phase of 42% acetonitrile in water at a flow rate of 0.8 ml/min.

column	t_0	ßNT	T	MT	tDES
retention time					
Hypersil	2.1	5.9	7.4	9.1	14.6
RP-18	2.9	12.9	16.3	21.5	28.3
Select B	3.0	13.0	16.3	20.3	29.8
capacity factor					
Hypersil		1.8	2.5	3.3	6.0
RP-18		3.4	4.6	6.3	8.6
Select B		3.4	4.5	5.9	9.1

both modifiers and different percentages of modifier. No optimum could be observed in the plate numbers at the different flow rates. Therefore, the flow was kept at 0.8 ml/min as recommended by the manufacturer.

In the second set of experiments three different HPLC columns were compared. The Hypersil ODS column was compared with two Superspher columns packed with RP-18 and RP-select B material, respectively. The three columns were compared with a mobile phase of 42% acetonitrile in water at flow rates of 0.8 ml/min. The results are summarised in Table 1. To the test set T was added, because it is a natural steroid and the other compounds should be separated from it. As expected, it eluted between ßNT and MT. It was intended to test the three columns with a mobile phase of 55% methanol in water, but the backpressure with the Superspher columns then became too high. According to Merck (G. Wieland, personal communication, 1995), this was normal with those columns and therefore, the comparisons were only performed with acetonitrile as organic modifier.

Some differences existed between the column materials. The Hypersil column contained fully endcapped octadecylsilane-covered spherical silica particles. A similar material was found in the Superspher RP-18 column. Yet, the Superspher RP-Select B column contained base-deactivated octylsilane groups and should be especially useful for the analysis of basic compounds. Several properties collected for the three columns are given in Table 2. The surface area of the Superspher material is higher than that of the Hypersil column as may be expected from the difference in particle diameter. Also, the surface coverage and the percentage carbon of the Superspher RP-18 column are higher

Table 2. Characteristics of the HPLC columns used for the experiments provided by the manufacturers on the column data sheets (1994) and on their Internet-pages (1998).

characteristic	Hypersil ODS	Superspher RP-select B	Superspher RP-18
length (mm)	150	244	244
diameter (mm)	4.6	4.0	4.0
column dead time (sec)*	83	95	108
measured t_0 (sec)**	104	144	144
N measured for ßNT	7,000	15,000	17,000
N measured for T	9,000	16,000	21,000
N measured for MT	8,000	15,000	19,000
N measured for tDES	7,000	19,000	20,000
particle size (µm)	5	4	4
surface area (m^2/g)	170	360	350
% carbon	10	11.5	21.0
surface coverage (µmol/m^2)	2.8	3.61	3.55

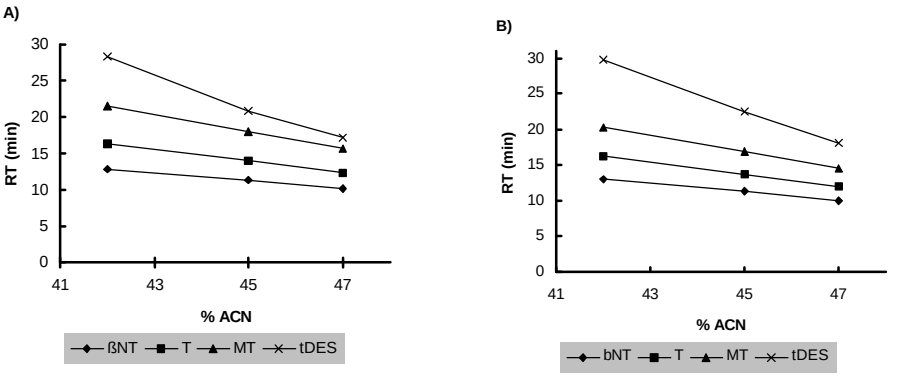
* column dead times provided on the column data sheet at a flow rate of 1.0 ml/min

** column dead time estimated in the isocratic HPLC system as the RT of unretained solvent at a flow rate of 1.0 ml/min

than those of the Hypersil ODS column, which contain the same C18 group. The Superspher columns were 10 cm longer than the Hypersil column. Also they contained 4 µm particles instead of 5 µm particles, which should result in better peak shapes. As expected, the RTs on the Superspher columns were longer and so were the estimated capacity factors (see Table 1). The peak shapes on the latter two columns were very good resulting in high resolutions, high plate numbers and high reduced plate heights (results not shown). Therefore, the Superspher columns were preferred for further work.

The mobile phase composition was optimised for the two Superspher columns at a flow rate of 0.8 ml/min to obtain retention times larger than 10 min to separate the steroids from polar matrix components expected in extracts of urine samples and to achieve a good separation of the steroids. The percentage acetonitrile was increased to reduce the RTs. The results are summarised in Figure 2. Increasing the percentage acetonitrile resulted in the expected reduction of RTs, but there were slight differences between the effects on the RTs of MT and tDES on the two columns. This resulted in significantly higher resolutions between MT and tDES on the RP-Select B column. As a

Figure 2. Retention times of three anabolic steroids and a stilbene on a Superspher RP-18 (A) and a Superspher RP-Select B (B) column with an isocratic mobile phase containing different percentages of acetonitrile.



consequence the resolution between T and MT was somewhat lower on the RP-Select B column, but the difference between the two columns was smaller than for the MT-tDES-pair. It was decided to use the RP-Select B column with a mobile phase consisting of 45% acetonitrile in water at a flow rate of 0.8 ml/min for further experiments.

The final experiment with the isocratic HPLC system concerned the linearity of the system. Standards were analysed in the range of 0.1-250 µg/ml or 2-5000 ng injected amount and peak heights were determined from the chromatograms. Calibration curves of the form y = a*x + b were constructed from the data. The results are summarised in Table 3. For ßNT the heights found with the 250 µg/ml standard were unacceptably low and were discarded as this concentration appeared to be beyond the linear range. Calibration curves for the other steroids were linear over the whole range studied. Detection limits were calculated from the calibration curves at three times the noise level. Negative values were obtained for ßNT and tDES, as the calculated intercept was larger than three times the noise level. However, it was assumed that the actual detection limits were similar to those of T and MT. Thus, the detection limits were in the range of 0.1-0.4 µg/ml or 2-7 ng injected amount.

Table 3. Calibration curves of the form y = a*x + b for three anabolic steroids and a stilbene in the final isocratic HPLC system. (x is the concentration in µg/ml; n is the number of data points; SEM is the standard error of the x-coefficient and the constant)

steroid	n	a	SEM	b	SEM	r^2	LOD (ng)
ßNT	66	0.00366	0.000014	0.00058	0.000448	0.9991	-3.1
T	69	0.00279	0.000009	-0.00030	0.000656	0.9993	2.2
MT	69	0.00246	0.000011	-0.00085	0.000594	0.9987	7.0
tDES	69	0.00187	0.000005	0.00008	0.000312	0.9996	-0.8

Gradient HPLC System

As the aim of the research was a multi-residue method, we wanted to be able to analyse more anabolic steroids with the same HPLC system. It was anticipated that a gradient HPLC system was needed to achieve a separation of all the steroids and to keep the analysis time within acceptable limits. Also, a multi-wavelength or diode array detector would be needed to detect all steroids in the same run. Therefore, research was continued with a System Gold HPLC pump and DAD detector, controlled with the System Gold Nouveau software. Initially, the RTs of 17 anabolic steroids were determined with the optimum isocratic HPLC system described in the previous section. Only those 17 could be tested as the other compounds became available at a later stage. The results are summarised in Table 4. The first steroid, Tb, eluted at an RT of 10.7 min, which would avoid interference by polar matrix components expected in extracts of urine samples. As expected, the RTs of the later eluting steroids were rather long, resulting in very broad peaks. However, peak shapes usually remained acceptable for the later eluting peaks, which is reflected in the theoretical plate numbers. Only AD co-eluted with tDES and HEX, whereas all other steroids were at least partially resolved. The Stan peak was extremely broad, resulting in a low plate number.

This preliminary experiment was done to select compounds for a test set for the development of the gradient. This test set should include the various structures among the anabolic steroids. Also, the earliest and latest eluting steroid (Tb and MED) were selected. E2benz was not considered in further studies, because it is an ester used for injection and will not appear in urine. It was later replaced by E2, which can be found in urine. tDES and HEX were included, because they eluted very near to each other. AD, a naturally occurring steroid, has no significant UV absorption at wavelengths larger than 220 nm [16] and will not interfere with the detection of relevant steroids. Therefore, it was not included in the test set and it was not used for further experiments. In view of the

Table 4. Retention times (min) and chromatographic characteristics of 17 anabolic steroids and related substances in the final isocratic HPLC system.

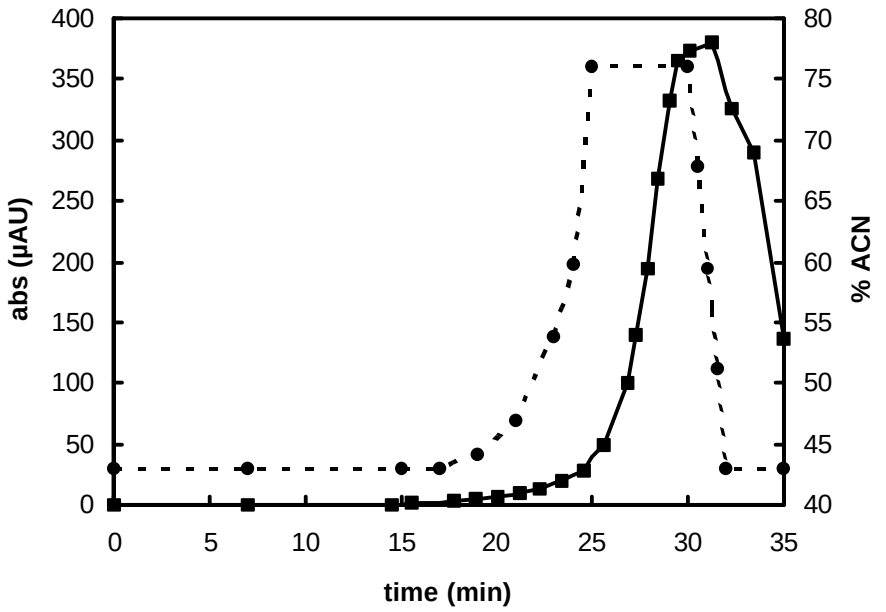
steroid	RT	k'	N	Rs	
t_0	1.4				
Tb	10.7	6.5	7000		
ßNT	11.7	7.3	6000	1.84	1.11
T	14.2	9.0	6000	3.66	1.24
EE2	15.3	9.8	5000	1.43	1.09
E1	16.3	10.5	5000	1.04	1.07
MT	17.4	11.3	6000	1.32	1.08
Zeara	19.2	12.5	5000	1.77	1.11
DE	21.6	14.3	4000	1.97	1.14
tDES	22.9	15.2	4000	0.92	1.06
AD	23.4	15.5	6000	0.39	1.02
HEX	23.5	15.6	5000	0.06	1.00
cDES	30.1	20.2	4000	4.10	1.30
P	37.6	25.5	8000	4.27	1.26
MP	44.9	30.7	7000	3.71	1.20
Stan	50.8	34.9	2000	1.81	1.14
MED	66.8	46.2	9000	4.48	1.32
E2benz	114.6	79.8	6000	11.10	1.73

above considerations, the test set consisted of Tb, E1, MT, Zeara, tDES, HEX, cDES, P and MED. The criteria used for the development of the gradient were:

a) The first compound should elute around 10 min from the start to minimise interference by polar components in extracts of urine samples.
b) The steroids should all elute within 35 min to keep the analysis time acceptable.
c) The compounds should be spread as evenly as possible over the available range to minimise interference among steroids.

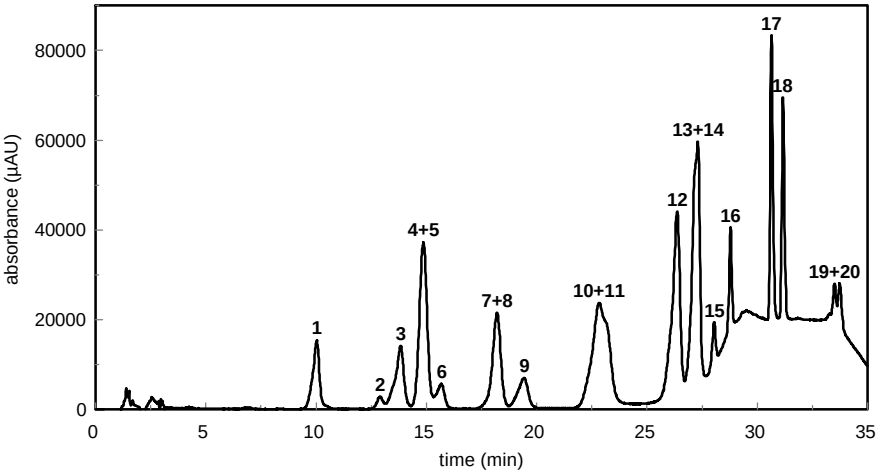
A test gradient from 40 to 67% acetonitrile in water was used and the seven gradient curves that can be produced by the Beckman software were all tested. A concave curve (curve 6 of the software) resulted in a nice spread of the test compounds and left room for the steroids that were not included in the test set. Yet, tDES and HEX were not separated with any of the gradients used. As MED did not elute within 35 min with the test gradient, the percentage of acetonitrile at the end of the gradient had to be increased to

Figure 3. Final gradient program for the separation of the anabolic steroids and related substances on a Superspher RP-Select B column. The straight line shows the pump program and the dashed line with the dots gives the detector signal measured at 190 nm.



76%. Also, the percentage at the start was increased slightly to 43% to make the earliest compound elute around 10 min. This resulted in the final gradient depicted in Figure 3. The RTs and chromatographic characteristics found for the anabolic steroids tested are given in Table 5 and a representative chromatogram is shown in Figure 4. The RTs of the steroids are spread quite evenly over the range of 10-35 min, but some peaks show overlap. In these cases the UV spectra of the compounds can be used to differentiate between substances. Only in the case of NT and ßNT the peaks coincide and the UV spectra are identical. However, this is not a problem as NT is a metabolite of ßNT [17].

Figure 4. Chromatogram of a standard solution containing 20 anabolic steroids and related substances at a concentration of 10 µg/ml (200 ng injected amount) using the final gradient elution program and recorded at 230 nm. The peak numbers refer to the steroids as given in Table 5.



The linear range was tested with calibration standards between 0.25 and 10 µg/ml or 5-200 ng injected amount. For E2, EE2, E1 and ClTac, no peak was observed with the 0.25 µg/ml standard. Stan was found to have only weak UV absorption and was very difficult to detect. LODs were calculated for the steroids at their specific detection wavelengths. They were calculated using the calibration curves at three times the noise level at that wavelength. For all steroids except the oestrogens, the LODs were below 5 ng injected amount. The oestrogens had slightly higher LODs of 5-10 ng injected amount.

Table 5. Retention times (min) and chromatographic characteristics of 21 anabolic steroids and related substances in the final gradient HPLC system. Peak numbers refer to the accompanying peaks in the chromatogram shown in Figure 4.

steroid	peak number	RT	k'	Rs		linear range (µg/ml)	LOD (ng)
t_0		1.4					
Tal	1	10.0	6.0				2
Tb	2	12.9	8.1	5.73	1.33	0.25-10	4
Zer	3	13.8	8.7	1.64	1.08		2
ßNT	4	14.8	9.4	1.63	1.08	0.25-10	1
NT	5	14.9	9.5	0.26	1.01		2
E2	6	15.7	10.0	1.28	1.05	0.5-10	8
EE2	7	18.1	11.7	3.48	1.17	0.5-10	9
T	8	18.2	11.8	0.11	1.01	0.25-10	1
E1	9	19.5	12.7	1.79	1.07	0.5-10	7
Zeara	10	22.8	15.0	3.96	1.19	0.25-10	1
MT	11	23.2	15.3	0.49	1.02	0.25-10	2
DE	12	26.4	17.5	4.83	1.15	0.25-10	3
tDES	13	27.2	18.1	1.53	1.03	0.2-7.5	0.5
HEX	14	27.3	18.2	0.43	1.01	0.25-10	0.5
ClTdiol	15	28.0	18.7	2.16	1.03	0.25-10	1
cDES	16	28.8	19.2	2.94	1.03	0.15-3	0.5
P	17	30.6	20.5	8.45	1.07	0.25-10	0.5
MP	18	31.2	20.9	2.35	1.02	0.25-10	1
Stan		31.8	21.3	2.04	1.02		> 600
MED	19	33.5	22.5	4.81	1.06	0.25-10	0.5
ClTac	20	33.7	22.7	0.80	1.01	0.5-10	3

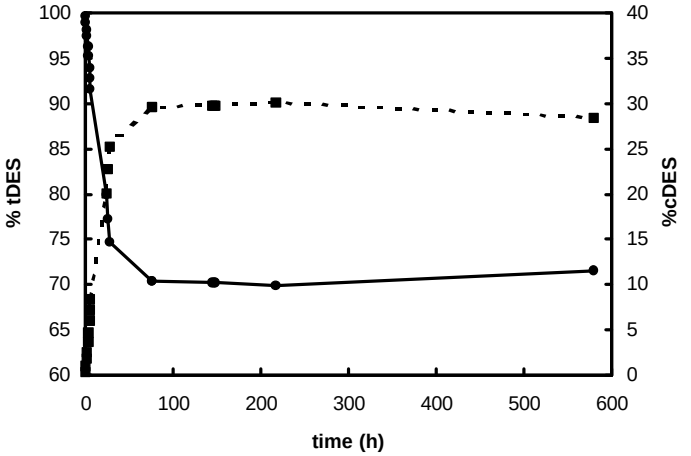
From Tables 4 and 5 it can be seen that EE2 and T, as well as Zeara and MT switched positions in the final gradient as compared to the results in the isocratic system. The RTs of the oestrogens, stilbenes and resorcylic acid lactones were found to vary more than the RTs of the other steroids. This is most likely due to the more polar character of the former oestrogenic compounds caused by the presence of a phenolic hydroxy group. Their retention behaviour will be more influenced by slight changes in the composition of the mobile phase. A possible solution for this problem would be to add a buffer to the

mobile phase. However, the use of corrected retention times (RTcs) made the use of a buffer unnecessary (see below). As the addition of a buffer to the mobile phase presents other problems, like crystallisation with increasing amounts of acetonitrile and the necessity of thorough washing, the use of RTcs was preferred.

Cis-Trans Isomerism of DES

It is known that tDES in solution isomerises quite easily to cDES [18,19]. As a result, the stock solution and calibration standards of tDES always showed a second peak in the chromatogram. To assess the speed of isomerisation and to determine the ratio in which the trans- and cis-forms occur in the calibration standards an isomerisation experiment was performed. In this experiment a 0.5 mg/ml solution of tDES was prepared and analysed immediately afterwards. Then, samples were injected at regular intervals to follow the appearance of the second peak. The results are summarised in Figure 5. During the first 10 hours the isomerisation took place rapidly, but then it levelled off to a plateau that remained more or less constant at a ratio of 72% tDES and 28% cDES. The UV spectrum of the second peak was similar to the published UV spectrum of cDES [20,21]. The ratio of 72:28 for tDES and cDES was used for the calculation of absolute detection limits.

Figure 5. Isomerisation of tDES to cDES. The percentage of tDES and cDES measured at different times during the isomerisation are given.



Identification of Compounds

The retention behaviour and the UV spectrum are important tools in the identification of the steroids. Although neither parameter on its own provides adequate selectivity, the combined information from the retention behaviour an the UV spectrum may provide enough power to allow unambiguous identification [22].

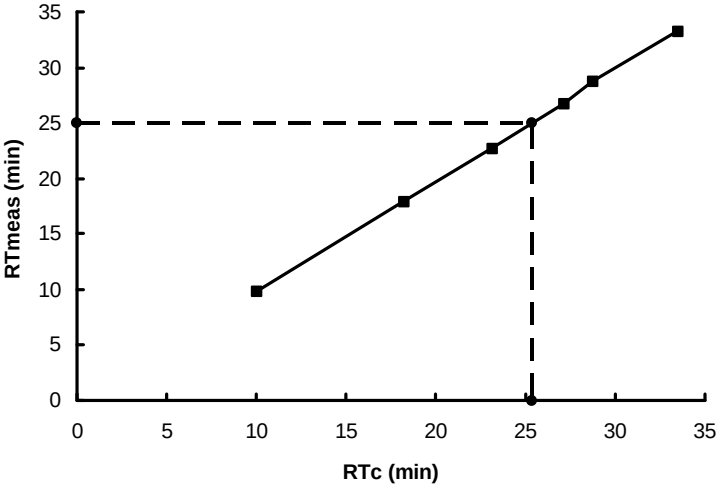
The RTs observed during our experiments were fairly stable, but inevitable variations occur due to small changes in mobile phase composition and room temperature. When the retention behaviour is to be used for identification of compounds a correction must be made to minimise variation [23].

It was decided to use a corrected retention time (RTc) for this purpose. This correction is made similar to the corrected hRf values used in the standard TLC systems for toxicological samples [24]. A calibration is made using a mixture of six reference substances of which the RTc is accurately known from repeated analyses. The substances selected as reference substances were Tal, T, MT, tDES, cDES and MED. The RT found in the actual experiment is plotted against the RTc to obtain a calibration graph (see Figure 6), which can be used for the correction of peaks observed in samples by interpolation. The RTc of unknowns can also be calculated using the following equation:

$$RTc(X) = RTc(A) + (RTc(B)-RTc(A))/(RT(B)-RT(A)) * (RT(X)-RT(A))$$

where A and B are the bracketing reference substances around unknown substance X. This procedure reduced the standard deviation of the retention parameter to 7-60% (average 26%) of its original value (see Table 6). It can also be seen from Table 6 that the RTs of the oestrogens, stilbenes and resorcylic acid lactones varied more between experiments than the RTs of the androgens and the progestagens, as was mentioned above. The use of RTcs reduced the standard deviations of all compounds to 0.10 min or less. Although they are still slightly higher for the oestrogenic compounds than for the androgens and progestagens, the value of 0.10 min is acceptable for calculations of similarity indices. Also, by using RTcs the standard deviation has become more or less constant over the whole RT range studied.

Figure 6. Example of a calibration graph for the correction of retention times.



The Beckman Gold Nouveau software provided the possibility to build a library of UV spectra and an algorithm is build into it to retrieve spectra and to calculate a similarity index (SI). However, the software cannot subtract a baseline file containing DAD data to correct for absorbances caused by the solvent used to make the gradient. As the acetonitrile used to make the gradient had a significant UV absorption between 190 and 230 nm, this seriously hampered the recognition of UV spectra of compounds that elute in the gradient part of the chromatogram (especially after 25 min). A procedure was developed to overcome this problem. The spectra of the detected peaks in a sample were exported as ASCII files and baseline subtraction could be performed manually in a spreadsheet program or by a home-made software program. A standard blank spectrum taken at a RT of 31 min can be used for subtraction, which can be corrected for the maximum absorption observed in the data-file at the same RT. For compounds eluting in the steep part of the gradient a percentage of the standard blank spectrum should be subtracted. The percentage to be used was calculated from the RT of the substance (see Figure 3). The resulting corrected spectra could be compared to corrected reference spectra, thus allowing a SI to be calculated [25].

Table 6. Retention times and corrected retention times of 20 anabolic steroids and related substances. The reference mixture and the steroids were analysed separately on the same day. Averages (avg) and standard deviations (sd) were calculated for both retention time (RT) and corrected retention time (RTc) (n=4).

steroid	RT (min)		RTc (min)	
	avg	sd	avg	sd
Tal	10.04	0.11	10.06	0.02
Tb	12.86	0.14	12.88	0.02
Zer	13.85	0.20	13.87	0.04
ßNT	14.75	0.15	14.76	0.03
NT	14.97	0.16	14.99	0.02
E2	15.57	0.21	15.59	0.06
EE2	18.02	0.27	18.04	0.10
T	18.17	0.20	18.19	0.01
E1	19.33	0.30	19.35	0.08
Zeara	22.67	0.35	22.69	0.09
MT	23.13	0.26	23.17	0.07
DE	26.24	0.28	26.27	0.08
tDES	27.06	0.19	27.08	0.03
HEX	27.24	0.18	27.26	0.04
ClTdiol	27.98	0.12	27.98	0.03
cDES	28.73	0.08	28.72	0.05
P	30.58	0.08	30.58	0.02
MP	31.09	0.08	31.10	0.02
MED	33.42	0.10	33.42	0.03
ClTac	33.64	0.09	33.66	0.04

The SI calculated from the UV spectrum was then combined with the SI calculated from the RT. The program used for these calculations has been designed in such a way that it produces a list of candidates for the unknown substance encountered, in decreasing order of similarity. The substance with the highest combined SI is the most likely candidate. In the event that two or more substances are listed at the top with little difference between their similarity indices, data obtained from other analytical methods, e.g. TLC, GC or MS may be introduced to provide further differentiation between the top candidates to the extent that only a single candidate remains [22].

Table 7. Peak ratios for 20 anabolic steroids and related substances at different wavelengths. Under 'percentage' the percentage of the height at that wavelength to the height at the specific detection wavelength of the steroid is given. 'LOD' represents the practical detection limits (ng) in extracts from calf urine at the different wavelengths. Empty boxes mean that the practical detection limit is larger than 200 ng or that there is interference by urine components.

steroid	percentage					LOD				
	192	230	242	280	350	192	230	242	280	350
Tal*	190	100	42	33	0	*	*	*	*	*
Tb	37**	17**	20	13	100		12	12	12	5
Zer*	160	100	37	36	0	*	*	*	*	*
ßNT	18**	63	100	2	0	95	5	5	48	
NT*	19	63	100	2	0	*	*	*	*	*
E2	830	100	8	48**	0	9	5		46	
EE2	100	13	2	6	0	11	22		22	
T	18**	63	100	2**	0	51	13	13		
E1	100	13	1	6	0	5			11	
Zeara	66**	103	100	47	0	12	10	10	10	
MT	19	64	100	2	0	94	5	5	94	
DE	220	100	75	15**	0	27	5	5	5	
tDES	380	91	100	33	0	19	4	4	4	
HEX	390	100	7	18	0	13	5	26	5	
ClTdiol	41	36**	100	25	0		12	5	12	
cDES	407	100	82	61	0	15	2	4	4	
P	32	71	100	1	0		6	6		
MP	36**	74	100	2	0	13	5	5	20	
MED***	29	13	16	100	1				5	
ClTac***	39	38	100	22	0					

* no experiments with urine samples could be performed with those compounds due to time restraints and therefore, no LODs can be given

** ratio may be affected in urine samples due to interference

*** detection at 192, 230 and 242 nm in urine samples is hampered by a co-eluting interference [26]

Comparison of the full spectrum of the unknown with the full spectrum in the library is to be preferred as this provides better identification power. However, at low analyte concentrations, the spectra obtained may become less suitable for identifications. As an alternative, peak ratios can be calculated at the five detection wavelengths. Reference values obtained in our experiments are given in Table 7.

Potentials of the Developed HPLC-DAD System

The HPLC-DAD system described here can be used for the analysis of extracts of urine samples from calf urine for the presence of residues of illegal anabolic steroids and related substances. This is being addressed in Chapter 2.2. Other possible uses include the analysis of seized preparations of illegal growth promoters. For the analysis of dosage forms simple extraction methods are sufficient. Tablets can be pulverised and extracted with chloroform [27] or methanol [10]. Oily preparations for injection can be extracted with methanol [10,11] followed by alkaline hydrolysis of the esters with potassium hydroxide [27]. Aqueous suspensions and emulsions can be diluted with methanol [10,11] and implants can be extracted with methanol [11] before analysis. Hydrolysis of esters of the steroids is necessary because some may not elute from the HPLC column within 35 min using the present gradient. Alternatively, the gradient may be adapted to allow analysis of intact esters by increasing the final percentage of acetonitrile to 100% [10,11].

Several other multi-residue HPLC systems have been developed for the analysis of anabolic steroids. Most of these used reversed phase columns [10-12,28,29], but separations on two normal phase columns have been reported as well [13]. In some of those studies only a limited number of compounds was used [13,28,29]. However, the methods were not intended for the analysis of urine samples [10-12,29] or they were intended as sample pre-treatment methods prior to immunological detection [28]. Three methods have been reported for the analysis of multiple anabolic steroids in illegal preparations [10-12]. Of those three studies only the last one may potentially be useful for the analysis of urine samples, as the first steroid elutes at 7 min. With the other two systems, the first compound elutes at a retention time of 1-2 min where polar matrix components will interfere with detection.

In conclusion, we have reported a method for the analysis and identification of 20 anabolic steroids and related substances. Identification in urine is based on the retention parameter plus the UV spectrum of the substance. Unambiguous identification of the 20 steroids tested is possible. The detection limits of the HPLC-DAD system are all below 10 ng injected.

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CHAPTER 2.2

SOLID PHASE EXTRACTION FOR MULTI-RESIDUE ANALYSIS OF ANABOLIC STEROIDS AND RELATED SUBSTANCES FROM CALF URINE USING C18 AND ALUMINA COLUMNS[1]

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Abstract

A solid phase extraction method for anabolic steroids and related substances in calf urine is reported, that is suitable as screening method for illegal growth promoters. Two types of sorbent were used: A reversed phase C18 material and a polar alumina material. After overnight enzymatic deconjugation, the 5-ml sample was first brought on the C18 column. This column was washed with 55% methanol in water and was then eluted with 95% acetone in water. The extract was directly brought on the alumina column. The run-through and an additional elution with 95% acetone in water were collected. The final extracts were analysed with an HPLC-DAD method described previously. The steroids are separated on a Superspher RP-Select B column with a gradient mobile phase consisting of acetonitrile and water. The method was found to be suitable for at least 19 illegally used anabolic steroids, having recoveries ranging from 65-110% at a spiking level of 25 ng/ml. Detection limits ranged from 0.6-80 ng injected amount or 1-160 ng/ml urine.

Introduction

Anabolic steroids and some related substances with comparable activities, all of which are here referred to as anabolic steroids, have been used as growth promoters

[1] A slightly modified version of this chapter has been submitted to The Analyst

during fattening of cattle for a long time [1,2]. This treatment may result in residues of the compound in the meat, which could be harmful for the consumer. From sports doping cases and therapeutic uses the anabolic steroids are known carcinogens and prolonged ingestion of larger doses disturb the endocrine balance, leading to a large number of side effects [3]. Although these effects are mainly expected when large doses are ingested, the consumption of meat that contained residues of oestrogenic compounds, has been suggested as the cause of breast enlargement in Italy [4] and precocious puberty in Puerto Rico [5]. Because of these risks the use of anabolic steroids as growth promoters in cattle was banned in the European Union in 1988 [6]. For the control of this ban, samples taken during fattening at the farm and after slaughter at the slaughterhouse are analysed for the presence of illegal growth promoters. Urine is the sample most often used for the analysis of the anabolic steroids. Analytical methods for human and equine urine [3,7] and for biological samples obtained from food-producing animals [8,9] have been reviewed. Most methods employ solid phase extraction (SPE) or immuno-affinity chromatography (IAC) for clean up of the sample and GC-MS for the detection of the steroids [3,7]. However, HPLC has been used for the analysis of anabolic steroids in preparations of illegal growth promoters [10-12] and as a clean up step for biological samples [13,14]. For SPE of steroids from urine mostly reversed phase sorbents have been used, including C2 [15] and C18 [16-25] bonded silica. Polar sorbents, like silicagel [20,24,26], alumina [22,24] and aminopropyl bonded silica [17,21,25], were used as additional clean up steps. IAC columns have been used alone [27] or in combination with C18 SPE [18] for the extraction of nortestosterone from urine. Other applications of IAC for the analysis of anabolic steroids in biological matrices have been reviewed [28].

Here, the development of a SPE method, capable of screening for a large variety of anabolic steroids and related substances used as growth promoters during fattening of cattle, in calf urine is reported. Two types of sorbent were used: A reversed phase C18 material and a polar alumina material. The method was found to be suitable for at least 19 illegally used anabolic steroids belonging to the androgens, oestrogens, progestagens, resorcylic acid lactones and stilbenes. The extracts were analysed with an HPLC-DAD method described previously [29].

Materials and Methods

Steroids and Related Substancs

The anabolic steroids used as reference substances were as follows. Methyltestosterone (MT) was from Serva (Serva Feinbiochemika GmbH, Heidelberg, Germany). Dienoestrol (DE), hexoestrol (HEX) and 17 -ethynyl oestradiol (EE2) were obtained

from Sigma (Sigma Chemical Company, St Louis, USA). Medroxyprogesterone (MP) was from Upjohn (Kalamazoo, Michigan, USA). BCR reference standards of zearalenone (Zeara), zeranol (Zer), taleranol (Tal), 19-17 -nortestosterone (NT) and 19-17 ß-nortestosterone (ßNT) were supplied by RIVM (Community Reference Laboratory/ Laboratory for Analytical Residue Research, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; further referred to as RIVM). Zeara, ß-trenbolone (Tb), 17ß-oestradiol (E2) and clostebol-diol (ClTdiol) standards were supplied by RIVM. Testosterone (T), ßNT, progesterone (P), medrogestone (MED), diethylstilbestrol (tDES and cDES) and oestrone (E1) were obtained from a local wholesaler.

All stock solutions were prepared in HPLC grade acetonitrile. Calibration standards were prepared in the range of 0.1-250 µg/ml, of which 20 µl were injected, by dilution of the stock solutions with either HPLC grade or gradient grade acetonitrile. The standard solution of tDES and cDES was found to contain a 72:28 mixture of trans- and cis-DES [29].

Some of the substance are sensitive to day light. The ratio of tDES to cDES changes under the influence of day light [30,31]. Standard solutions should, therefore, be kept in the dark.

Other Chemicals

Water was demineralised in house and when it was used for HPLC it was purified with a Maxima ultrapure water instrument (Elga, obtained from Salm en Kipp BV, Breukelen, The Netherlands). Methanol, acetone, and n-hexane were analytical grade (Merck KGaA, Darmstadt, Germany). Acetonitrile for HPLC (Labscan, Dublin, Ireland) was used for the preparation of stock solutions of the reference standards. Acetonitrile, gradient grade for chromatography (Merck), was used for mobile phases, for the dilution of calibrations standards and for the redissolution of samples. Ethyl acetate was analytical reagent grade (Labscan). Fuming hydrochloric acid (37%) and anhydrous sodium acetate were analytical grade from Merck. Aluminium oxide 90, active, neutral, activity I, particle size 0.063-0.200 mm (70-230 ASTM) for preparative chromatography was from Merck. Suc d'Helix Pomatia was from Sepracor/Biosepra SA (Villeneuve-la-Garenne, France)

Prepared Solutions

Concentrated hydrochloric acid (3.45 ml) was diluted with 6.55 ml demineralised water to obtain 4 M hydrochloric acid. The 2 M acetate buffer (pH 5.2) was made by

dissolution of 1.64 g sodium acetate in about 7 ml demineralised water, adjustment of the pH to 5.2 with 4 M hydrochloric acid and making up of the volume to 10 ml with demineralised water. For 55% methanol in water (v/v) and 95% acetone in water (v/v) appropriate amounts of the organic solvent and demineralised water were mixed. Solvent A of the mobile phase (40% acetonitrile in water v/v) was prepared by mixing 400 ml gradient elution grade acetonitrile with 600 ml demineralised and purified water. Solvent B was gradient grade acetonitrile. Both mobile phase solvents were degassed using vacuum and sonication prior to use.

Urine Samples

Blank calf urine samples and reference blank bovine urines (5 ml lyophilised, codes bov01-bov20) were provided by RIVM. Samples were stored at -18 °C until analysis to prevent decomposition.

SPE Columns

LiChrolut® RP-18 columns for solid phase extraction with 200 mg sorbent, and Extrelut® 20 pre-packed columns for extraction of lipophilic compounds from aqueous solutions (20 ml samples) were from Merck. Neutral alumina columns were home-made using cleaned standard 3-ml polypropylene SPE columns (id 9 mm): 1.00 g of neutral alumina was dry-packed between two cleaned PTFE frits.

Equipment

A PHM 62 standard pH meter with combined pH electrode GK2501C was from Radiometer (Copenhagen, Denmark). The Megafuge 1.0 was obtained from Heraeus Sepatech GmbH (Osterode, Germany) and the vortex mixer from Wilten & Co BV (Etten-Leur, The Netherlands). The waterbath (Gebr. Haake, Berlin, Germany) was operated at 37 °C. The SPE-column processing system was a Baker SPE 12-g vacuum manifold (Mallinckrodt Baker BV, Deventer, The Netherlands). A Bransonic ultrasonic cleaner model B2210-E-MT was obtained from Bransonic (Bransonic Ultrasonics Corporation, Danbury, CT, USA). Vacuum and nitrogen were available through in-house facilities.

HPLC System and Conditions

The HPLC pump was a System Gold® 126 solvent module (Beckman Instruments Inc., Mijdrecht, The Netherlands) equipped with a System Gold® 168 DAD detector (Beckman). The pump and the detector were controlled with the Gold Nouveau Chroma-

tography Data System® version 1.0 (Beckman) run on an IBM personal computer 330p100 (Beckman) equipped with a HP deskjet 510 printer (Hewlett Packard, Amsterdam, The Netherlands).

The HPLC column was a LiChroCART® 250-4 HPLC cartridge, containing Superspher® 60 RP-select B material, 250x4 mm (Merck), protected by a LiChroCART® 4-4 guard column with LiChrospher® 60 RP-select B material, 4x4 mm (Merck). The injector was a Rheodyne 7725i injector equipped with a 20 µl sample loop (Rheodyne, Cotati, CA, USA).

The flow was set at 0.8 ml/min. The gradient was made up from 40% acetonitrile in water (v/v) (solvent A) and gradient grade acetonitrile (solvent B). The solvent module was programmed to deliver the following gradient:
0-5 min: 95% A and 5% B (43% acetonitrile in water (v/v))
5-25 min: gradient from 95% A to 40% A with curve 6
25-30 min: 40% A and 60% B (76% acetonitrile in water (v/v))
30-32 min: linear gradient from 40% A to 95% A (curve 0)
32-45 min: restabilise at 95% A and 5% B (43% acetonitrile in water (v/v))

The DAD-detector was programmed to collect data for 35 minutes from the start of the run. An autozero scaling was performed at the start of each new run. The scan range was 190-400 nm. Data were collected at a rate of 2 Hz. Readings were performed at 192, 230, 242, 280, or 350 nm (bandwidth 4 nm) depending on the steroid studied. For routine operation the software can be programmed to collect data at those five wavelengths (multichromatogram mode). Spectra were saved for detected peaks in this mode. Detection wavelengths for the quantitation of steroids were:
192 nm: E2, EE2, E1
230 nm: DE, HEX, cDES, Tal, Zer, sometimes E2 (all steroids can be detected at this wavelength)
242 nm: T, NT, ßNT, MT, tDES, P, MP, ClTac, ClTdiol, Zeara
280 nm: MED
350 nm: Tb

Methods

Final Extraction Procedure

The pH of a 5-ml urine sample was adjusted to 5.2 with 4 M hydrochloric acid. Then 1 ml 2 M acetate buffer pH 5.2 and 20 µl Suc d'Helix Pomatia were added and the mixture was incubated overnight at 37 °C. Thereafter, the sample was centrifuged for 12 min at 4000 rpm to remove particles that could block the SPE column. The C18 column

was conditioned consecutively with 2 ml methanol and 2 ml water using slight vacuum (< 5 in Hg), followed by the application of the hydrolysed and centrifuged sample. Then the column was washed with 2 ml 55% methanol in water under slight vacuum. After drying for 5 min under full vacuum the steroids were eluted with 3 ml 95% acetone in water under slight vacuum. The neutral alumina column was conditioned consecutively with 5 ml hexane and 5 ml acetone. The extract of the C18 column was then applied to the column and the run-through was collected. The column was dried briefly under vacuum and was then eluted with 2 ml 95% acetone in water. For this part of the procedure no vacuum was needed except for drying. The run-through and the extract were combined and were evaporated to dryness under nitrogen at 37 °C. The residue was redissolved in 200 µl acetonitrile and 20 µl was injected into the HPLC system described above.

In all cases care should be taken to prevent the columns to run dry during the conditio-ning, sample application and washing steps.

Validation of the Final Procedure

In the first validation experiments calf urine samples spiked at 5 different levels were extracted together with a blank sample. The spiking levels were 10, 25, 50, 100 and 200 ng/ml, respectively, for which 10-50 µl of a suitable spiking solution were used. The samples were then hydrolysed, extracted and analysed according to the procedures described above. Absolute recoveries were calculated using calibration standards in the range of 0.25-10 µg/ml (5-200 ng injected amount). If necessary, a correction was made for endogenous peaks in the extract of the blank sample. Calibration curves for extracted samples were constructed from the results. These calibration curves were used for the calculation of precision and accuracy in the experiments described below.

In the second validation experiment the repeatability was determined at a spiking level of 10 ng/ml. Four samples were spiked at the 10 ng/ml level with 20 µl of a 2.5 µg/ml solution of the respective steroid. They were then hydrolysed, extracted and analysed according to the procedures described above, together with a blank sample. Absolute recoveries were calculated using the respective calibration standards in the range of 0.25-10 µg/ml (5-200 ng injected amount). The repeatability was calculated as the relative standard deviation of the recoveries obtained for the four spiked samples. Accuracy and precision were calculated with the calibration curves for extracted samples. The accuracy was defined as the relative difference between spiked level and level found. The precision was calculated as the relative standard deviation of the levels found for the four spiked samples.

In the third validation experiment the repeatability and reproducibility were determined at a spiking level of 25 ng/ml. Two samples were spiked at the 25 ng/ml level with 25 µl of a 5 µg/ml solution of the respective steroid. They were then hydrolysed, extracted and analysed according to the procedures described above, together with a blank sample. The same experiment was performed on five different days. On the last two days, a new lot of C18 SPE columns was used. Absolute recoveries were calculated using the respective calibration standards in the range of 0.25-10 µg/ml (5-200 ng injected amount). The repeatability and reproducibility were calculated from one-way ANOVAs [32]. The reproducibility was defined as the relative standard deviation of the recoveries obtained on different days. Accuracy and precision were calculated with the calibration curves for extracted samples. The accuracy was defined as the relative difference between spiked level and level found. The precision within and between days was calculated from one-way ANOVAs [32]. To assess whether the use of the new lot of SPE columns had affected the results a Mann-Whitney rank sum test was performed [32].

Determination of False Positives and False Negatives

In the final validation experiment the number of false positives and false negatives was determined. For this experiment a reference bank of 20 blank bovine urine samples was used. For each sample in the bank, a blank and a spiked sample were analysed. Samples were spiked at the following levels using 50 µl of a suitable spiking solution: 9.2 ng/ml Tb, 4.8 ng/ml ßNT, 30.4 ng/ml E1, 9.9 ng/ml Zeara, 10.7 ng/ml DE, 7.7 ng/ml tDES, 4.8 ng/ml ClTdiol, 3.1 ng/ml cDES, 3.1 ng/ml MP, 5.2 ng/ml MED. The extracts were analysed together with a standard solution, which was used to estimate the retention times of the other steroids and as an indication for the recovery of the steroids in the spiked samples. When the recovery of the spiked steroids was 100%, the peak found in the spiked sample would be equal to the peak in the standard. Chromatograms were studied for the presence of peaks at 192, 230, 242, 280 and 350 nm. The criteria for peak detection were: a) The peak had to be within a reasonable distance (± 1.5 standard deviation) from the expected retention time of the steroid, and b) The peak height had to be larger than three times the noise at that wavelength. The UV spectra of detected peaks in the spiked urine samples of calves and the young bull were exported as ASCII files and correlation coefficients between reference spectrum and spectrum in the sample were determined as a measure of the similarity between the two spectra [33].

Calculation of Results

All results were calculated using peak heights at the specific detection wavelengths of the steroids, as given in the section 'HPLC System and Conditions' above. For all statistical test a significance level of 5% was used.

The limits of detection (LODs) for the 19 anabolic steroids for the HPLC-DAD system for calibration standards and for analytes extracted from calf urine were calculated using calibration standards in the range of 5-200 ng (injected amount). From the chromatograms the average noise was determined for both calibration standards and urine samples. The calculated amount at three times the noise level for calibration standards was taken as the LOD of the HPLC-DAD system. The average blank signal of four calf urine samples plus three times the noise level for urine samples or the standard deviation of the four blank signals, depending on the number of positive blanks, was used to calculate the LODs for steroids extracted from calf urine.

Results and Discussion

Development of the Procedure

The development and characteristics of the HPLC-DAD system were already given in Chapter 2.1 [29]. The system was originally set up for 21 anabolic steroids and related substances. However, stanozolol could not be detected at low levels due to its weak UV absorption. Also, the stanozolol peak observed was rather broad. During the first studies with urine samples it became clear that a matrix interference co-eluted with clostebol acetate. As it was not possible to get rid of this peak, clostebol acetate was not included in further studies. However, the acetate ester is not excreted as such and the metabolite ClTdiol can be determined [34]. Initial studies were performed with 16 anabolic steroids. Tal, Zer and NT became available in a later stadium and they were not used in all experiments.

The first step in the analytical procedure is the hydrolysis of conjugates of the steroids and their metabolites [35,36]. Suc d'Helix Pomatia was used, because it is suitable for both glucuronides and sulphates. Deconjugation for 2 hours at 50 °C may be performed, but overnight hydrolysis was found to result in cleaner extracts (R.K. Vermeulen, RIVM, personal communication, 1996).

A C18 SPE column was used as a first clean up step. Yet, it rapidly became apparent that these columns, which are frequently used to extract drugs from human urine, had difficulties in adequately cleaning up bovine urines. The resulting extracts showed a considerable number of endogenous peaks. In order to improve the results the percentage of methanol in the wash step was optimised to get the cleanest possible extract with a

good and reproducible recovery of the steroids. Also, a wash step with hexane, performed immediately after the wash with the methanol-water mixture, was evaluated. However, significant losses of the steroids were observed already with small volumes of hexane. The elution solvent and elution volume were also optimised. Ethyl acetate appeared to be a good elution solvent as was found before by others [17,21].

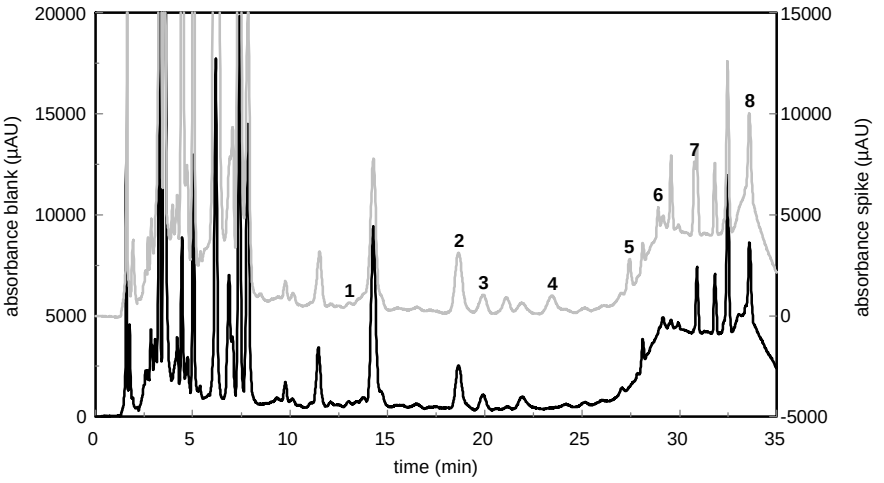
Although all the steps in the C18 SPE procedure were optimised, the extracts of the urine samples still contained a lot of interfering compounds. Therefore, an additional clean up step with an alumina SPE column was evaluated. The extract resulting from the C18 SPE step was brought on the column without evaporation. The steroids should pass through the column unretained, whereas matrix components are being retained. Neutral alumina was found to result in the cleanest extracts with a good recovery for most of the steroids in the run-through. However, Zeara was retained by the alumina when organic solvents were used for sample application and elution. In the literature a solution of 95% acetone in water was suggested as a good elution solvent for oestrogenic compounds from alumina columns [37-39]. This solvent was further evaluated. It was also found to be equally suitable as elution solvent for the C18 SPE column as ethyl acetate. Therefore, the ethyl acetate, which had previously been used as elution solvent for the C18 SPE column, was replaced by 95% acetone in water. In the end, after the collection of the run-through, the alumina column was eluted with 2 ml 95% acetone in water, which resulted in a recovery of Zeara of about 80%.

The resulting final procedure given in the methods section was further evaluated with urine samples from calves and cows. Whereas extracts of calf urine were relatively clean, extracts of cow urine were relatively dirty and many interfering peaks were observed in the chromatograms (see Figure 1). This problem was expected as urine from adult animals is known to contain metabolites of endogenous steroids [40] and other potentially interfering substances.

Validation

Recoveries of the anabolic steroids were determined at spiking levels of 10, 25, 50, 100 and 200 ng/ml urine. The results are summarised in Table 1. Some compounds could not be detected at the 10 ng/ml spiking level either because of interference (T, cDES, P) or because this level resulted in amounts of steroid in the extract below the detection limit (EE2, E1). Interfering peaks also caused problems with the determination of the recovery of T, DE and P at other spiking levels. For the other steroids, the recovery remained fairly constant over the concentration range studied. The results of this experiment were used to construct calibration curves of the steroids extracted from urine

Figure 1. Chromatograms of extracts of representative urine samples obtained with the final SPE procedure recorded at 230 nm. The upper panel shows a blank (lower trace) and spiked (upper trace) calf urine sample. The sample was spiked at the 25 ng/ml level with Tb (1), T (2), E1 (3), Zeara (4), tDES (5), cDES (6), P (7) and MED (8). In the lower panel a blank cow urine sample is shown. Note the scale differences between the two panels. Not all spiked analytes can be detected at this level at 230 nm.



samples, which were used to calculate accuracy and precision in later experiments. In Table 2 and Figure 2 the calibration curves calculated for calibration standards and for extracted spiked samples are compared. Both curves had high correlation coefficients indicating that the curves were linear. Generally, the correlation coefficient for extracted samples was somewhat lower than for the calibration standards. The constant for the calibration standards was in most cases small and was never significantly different from zero. For the extracted samples, sometimes rather large, negative constants were obtained. In these cases an interference was observed in the blank samples. When the x-

Table 1. Absolute recoveries (%) of 16 anabolic steroids and related substances from calf urine samples spiked at different levels (ng/ml, n=1, nd = not detected)

steroid	recovery at spiking level				
	10	25	50	100	200
Tb	98	91	86	83	90
ßNT	88	81	81	85	104
E2	64	44	54	90	120
EE2	nd	nd	55	78	109
T	nd	51	50	70	96
E1	nd	29	51	57	97
Zeara	96	84	81	64	78
MT	92	103	87	100	106
DE	49	69	56	58	90
tDES	136	102	82	72	86
HEX	90	85	69	75	105
ClTdiol	123	101	89	97	110
cDES	nd	124	104	90	107
P	nd	28	57	71	95
MP	97	94	85	97	96
MED	34	70	72	74	90

coefficients of both curves are compared an indication is obtained for the recovery from extracted samples. Generally, the x-values of both curves are similar, indicating near-quantitative recoveries.

Then, the repeatability was determined at the 10 ng/ml spiking level (n=4). The results are summarised in Table 3. As this spiking level is below the detection limit of the oestrogenic compounds (E2, EE2 and E2), they were either not detected or unrealistic recoveries with very large standard deviations were observed. In this urine sample, matrix compounds interfered with the detection of T, MT, cDES and P resulting in too large or too small recoveries and/or large standard deviations. Accuracy and precision were calculated using the calibration curves for steroids extracted from urine samples. For the steroids for which interferences caused problems in one or both of the experiments large differences from the spiked amounts were obtained as reflected by large deviations under accuracy. For HEX, ClTdiol and MED differences in the recovery

Table 2. Comparison of the calibration curves (y = a*x + b; y: peak height (µAU), x: ng injected in case of 100% recovery) for the anabolic steroids and related substances calculated for calibration standards and for extracted spiked samples. For the calibration curve for the standards four data points per concentration were used. For the calibration curve for extracted samples one data point per spiking level was used.

steroid	standards			samples		
	b	a	r^2	b	a	r^2
Tb	37	75	0.9981	3	68	0.9981
ßNT	-150	190	0.9969	-460	190	0.9906
E2	390	220	0.9871	-1500	260	0.9741
EE2	25	140	0.9983	-1320	240	0.9716
T	77	210	0.9916	-940	140	0.9781
E1	340	220	0.9961	-1700	210	0.9627
Zeara	-20	100	0.9983	-94	83	0.9897
MT	-57	120	0.9977	-160	130	0.9978
DE	460	220	0.9520	-940	160	0.9657
tDES	120	240	0.9912	-350	200	0.9878
HEX	120	290	0.9872	-880	240	0.9755
ClTdiol	-31	160	0.9963	-230	160	0.9944
cDES	170	310	0.9903	-800	320	0.9780
P	110	400	0.9981	-3000	390	0.9832
MP	-230	340	0.9969	-160	330	0.9990
MED	7.5	340	0.9977	-680	300	0.9938

obtained in the two experiments explained the large difference found under accuracy. Repeatability and precision were in most cases similar. However, for T, cDES and P the precision was much better. In these cases, where interference by matrix components made determination of the recovery difficult, the use of a calibration curve for the steroid extracted from urine was very useful.

In the third experiment, the repeatability and reproducibility were determined at the 25 ng/ml spiking level (n=2 on 5 different days). The results are summarised in Table 4. This spiking level is near the detection limit of the oestrogenic compounds (E2, EE2 and E1) and an interfering peak was observed around the retention time of T and EE2. This resulted in low recoveries and large standard deviations. For E1 the data of day 5 were excluded, because a very large interference peak was observed in the blank, which was

Figure 2. Calibration curves of Tb (A) and E2 (B) for calibration standards (straight line) and after extraction from urine samples (dashed line, dots indicate the signals obtained for the different samples).

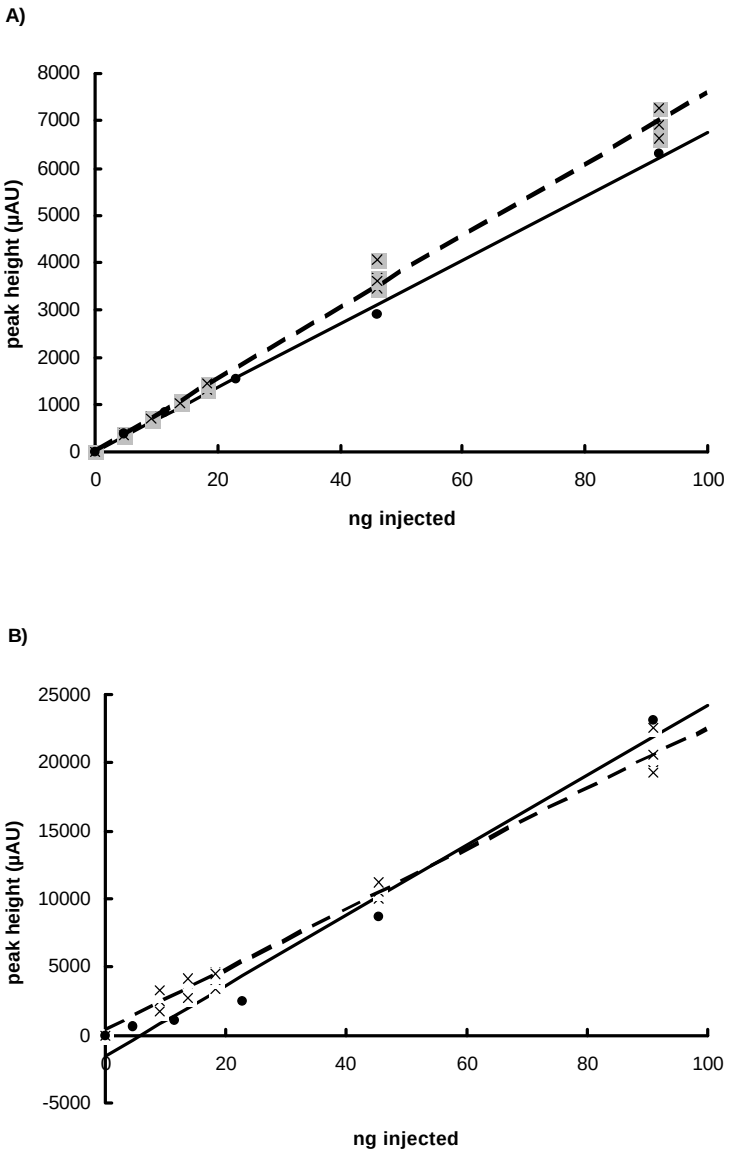


Table 3. Repeatability of the extraction of 16 anabolic steroids and related substances spiked at the 10 ng/ml level from calf urine (n=4; nd = not detected).

steroid	recovery (%)	repeatability (%)	accuracy (%)	precision (%)
Tb	117	7	20	8
ßNT	105	9	41	6
E2	153	73	182	33
EE2	nd		nd	
T	42	72	61	19
E1	96	41	137	19
Zeara	91	10	-4	12
MT	130	2	46	2
DE	109	6	107	4
tDES	84	7	7	6
HEX	121	5	104	3
ClTdiol	58	11	-17	7
cDES	133	51	267	20
P	128	9	158	5
MP	108	2	11	2
MED	111	12	67	10

larger than the peaks observed in the spiked samples. The repeatability was in most cases similar to that found with the samples spiked at the 10 ng/ml level. The day-to-day reproducibility was in most cases somewhat worse. The accuracy was generally better at the 25 ng/ml level than at the 10 ng/ml. For E2, T and EE2 the accuracy was very good, despite the fact that the recovery was low. Here again the use of a calibration curve for the steroid extracted from urine was very useful. The large value under accuracy found for E1 can be explained from the fact that the spiking level is near the detection limit. For P and cDES, also, a large deviation was found under accuracy. This can be explained by the presence of the matrix peak in the sample used for the construction of the calibration curve extracted from urine. As observed for the 10 ng/ml level, the precision values were similar to those for the repeatability and the reproducibility. The large standard deviations found for T are caused by the matrix interference.

Table 4. Repeatability and reproducibility of the extraction of 16 anabolic steroids and related substances spiked at the 25 ng/ml level from calf urine (duplicate analyses were performed on five different days). Rec is the absolute recovery (%), CV1 the repeatability (%), CV2 the reproducibility (%), Acc the accuracy (%), P1 the precision within-days (%) and P2 the precision between-days (%).

steroid	Rec	CV1	CV2	Acc	P1	P2
Tb	93	4	19	4	4	20
ßNT	88	16	3	1	13	10
E2	64	98	199	8	48	105
EE2	30	132	371	0.3	32	75
T	65	35	38	5	40	71
E1*	94	34	17	81	21	12
Zeara	73	8	44	-3	8	34
MT	102	10	11	2	9	9
DE	70	8	21	6	5	17
tDES	73	6	32	-13	5	26
HEX	101	14	15	24	12	15
ClTdiol	109	14	23	6	14	22
cDES	92	9	25	59	6	19
P	90	2	19	53	2	12
MP	93	8	13	-2	8	4
MED	79	3	15	12	3	14

* data for day 5 were excluded because an exceptionally high value was observed in the blank sample.

For the reproducibility experiment at the 25 ng/ml spiking level two different lot numbers of C18 SPE columns were used. Mann-Whitney tests were performed to assess whether there were significant differences between the recoveries obtained with the two lots. This was the case for E2, Zeara and ClTdiol, where the recoveries of day 1-3 were significantly higher, and for EE2 and MP, where the recoveries of day 4 and 5 were significantly higher. The results for E2 and EE2 can be explained by negative recoveries found for several samples due to the interfering matrix peak. For ClTdiol, the results on day 1, 2, 3 and 5 were similar and those on day 4 were worse for unknown reasons. For MP and Zeara, the difference between the two datasets appears to be real, but the effect of the different lots on the two steroids is different. No explanation for this behaviour was

found. Since the above findings indicate that lot-to-lot variabilities may occur for at least some steroids, it is recommended that recoveries be checked before a new lot number of SPE columns is used for routine samples.

Determination of False Positives and False Negatives

The number of false positives and false negatives was determined with 20 reference blank bovine urine samples. This bank contained representative blank urine samples from various kinds of bovine animals. The samples were subdivided in four groups: cows (n=9), bulls (n=4), calves (n=4), young animals (n=3). The calves were all younger than 6 months. The category young animals consisted of samples of a young cow, a young bull and a heifer, all aged between 6 and 12 months (L.A. van Ginkel, RIVM, personal communication, 1997). Although it was determined in earlier experiments that the method was not very suitable for the analysis of cow urine, it was decided to analyse all 20 blank samples to see to what extent the method may be useful in the analysis of adult animals.

False positives were determined with blank urine samples. The chromatograms were checked for the presence of peaks around the expected retention times of the steroids. The criteria for peak detection were: a) The peak had to be within a reasonable (within 1.5 standard deviation) distance from the expected retention time of the steroid, and b) The peak height had to be larger than three times the noise. False negatives were determined for 10 steroids spiked in the samples around their respective detection limits. The results of these experiments with calf urine samples are summarised in Table 5. Only the results at the specific detection wavelength of the steroids are given here. Using the criteria given above peaks of endogenous steroids detected in blank urine samples will be marked as false positives. In these cases normally an action limit is used, above which a sample is marked as positive. As it was intended to combine the information of the retention time and the UV spectrum, we wanted to inspect all UV spectra of detected compounds and not only of the compounds that exceeded the action limits. Therefore, it was decided to use the criteria given above instead of action limits.

Calf urine resulted in clean extracts (see Figure 1). False positives were observed for several steroids, but generally the peaks were only small. The peak that co-eluted with Tal was always smaller than 2 mAU and it had a different UV spectrum with a broad maximum around 250 nm. Around the retention time of Zer two larger peaks eluted. The first had a UV spectrum that was recognised as originating from an oestrogen. The second peak had a maximum at around 255 nm, but no identity could be proposed as yet.

Table 5. Number of false positives and false negatives for the anabolic steroids and related substances in calf urine samples (n = 4). False positives were determined in blank urine samples and false negatives for 10 steroids were determined with blank urine samples that were spiked around the detection limits of these steroids.

steroid	false positives	false negatives
Tal	3	
Tb*	0	4 (0)
Zer	2	
ßNT	0	3
NT	0	
E2	0	
EE2	2	
T	4	
E1	0	0
Zeara	0	2
MT	1	
DE	4	0
tDES	1	0
HEX	0	
ClTdiol	0	1
cDES	3	0
P	3	
MP	0	0
MED	4	0

* Tb was spiked below the detection limit, but a peak was visible at the proper retention time in all samples as indicated between brackets.

The presence of those peaks made detection and recognition of the UV spectrum of Zer rather difficult. Around the retention times of T and EE2 a peak with maxima of 207 and 255 nm was observed. In two calf urine samples, the UV spectrum of the peak eluting at this retention time was recognised as that of T. A peak was present around the retention time of E1 with a maximum of 265-270 nm. This peak was quite large, but E1 could be detected at 192 nm although recognition of the spectrum was a problem. In one calf urine sample a large interfering peak was observed around the retention time of MT. This peak

had maxima at 243 and 287 nm. The peak eluting around the retention time of DE was always smaller than 2 mAU. From 26 min on, the spectra of interferences generally showed maxima around 250 and 285 nm, which were always smaller than 3 mAU.

In calf urine samples, the number of false negatives was limited. With regard to Tb, it later became clear that this compound had been spiked below the detection limit. However, a small peak was visible at the proper retention time in all calf urine samples. Similarly, ßNT and Zeara were detected in all four calf urine samples, but only in some cases the peak was larger than three times the noise level.

The number of false positives and false negatives found in adult urine samples was quite large. In cow urine, more than 5 samples had peaks at the retention times of nearly all steroids except Tb, ßNT and E2. For bull urine the situation was slightly better. No false positives were found for Tb, ßNT, NT, E2 and HEX and only one sample contained a peak with the same retention time as E1 and P. In the category young animals, the samples of the young cow and the heifer showed a peak pattern similar to that of cow urine with many false positives. Only for Tb, E2, EE2 and E1 no false positives were observed. The urine of the young bull was rather clean with false positives only for Tal, Zer, T, P and MED. This pattern is similar to that observed for calf urine. Due to the large number of false positives in the urine samples of adult animals, the number of detected false negatives was only small. However, ßNT showed only a limited number of false positives, yet it was not detected in 16 of the 20 spiked samples. Only in 5 of the negative samples a peak was detected that was smaller than three times the noise level.

When a peak was detected at the expected retention time of a natural steroid in a blank sample, the UV spectrum was compared to the library spectrum for that compound. The concentration of the natural steroids was determined (Table 6), when the spectrum matched the library spectrum as indicated by a high similarity index (larger than 0.8) calculated by the DAD software. In eight of the twenty samples a natural steroid was detected. T was detected at a concentration of about 70 ng/ml in two calf urine samples and E1 and E2 were detected in five and one cow urine sample, respectively. The levels of E1 were quite variable and ranged from 70 to 540 ng/ml.

The recognition of steroids with the help of UV spectra was discussed in Chapter 2.1 [29]. However, problems encountered with urine samples were not treated there. For the spiked calf urine samples and the spiked urine sample of the young bull, the UV spectra were compared to library spectra and they were visually inspected. As the steroids were spiked around their respective detection limits, spectra were distorted due to noise.

Table 6. Natural steroids detected in blank bovine urine samples. In the column labelled 'SI spectrum' the similarity index generated by the DAD software is given as an indication of the agreement between the UV spectrum of the peak in the extract and the UV spectrum in the library. (conc = estimated concentration)

sample number	type of animal	steroid	conc (ng/ml)	SI spectrum
bov01	cow	E1	540	0.9878
bov04	cow	E1	290	0.9677
bov05	calf	T	69	0.9505
bov08	cow	E1	72	0.9407
bov09	cow	E1	73	0.9257
bov10	cow	E2	330	0.9870
bov12	cow	E1	108	0.9654
bov20	calf	T	65	0.9459

Therefore, the correlations between reference spectrum and spectrum in the sample were lower (in almost all cases $r < 0.8$). However, visual inspection of the spectra showed that expected peak maxima were present in most cases. In some spectra, other UV maxima were observed caused by matrix components that eluted around the retention time of the steroid. Usually, the interfering peaks were small than 2-3 mAU and will only give problems when low amounts of analyte (5-20 ng, depending on retention time and spectrum of the steroid) are present in the sample. In these cases recognition of the UV spectrum will always be difficult and further confirmatory analysis is necessary.

Some additional notes are to be made. The pH value of the calf urine samples was generally lower than the pH of samples from the other animals (8.3 vs 8.6, $p < 0.05$). Also, much less 4 M hydrochloric acid was needed for calf urine to adjust the pH to 5.2 (3 drops vs 14 drops for adult urines, $p < 0.001$). Therefore, calf urines are better adjusted with 0.1 M hydrochloric acid. The extracts of calf urine samples were colourless, whereas bull urine resulted in yellowish green coloured extracts and cow urine in pink to purple coloured extracts. There was only one exception to this rule. Sample bov11 from a cow resulted in a yellowish brown coloured extract. The samples of the young cow and the heifer behaved similarly to the samples of cow urine and the extracts were coloured pink. The sample of the young bull, however, was in all respects similar to the calf urine samples.

Detection Limits

The LODs for the 19 anabolic steroids and related substances for the HPLC-DAD system and for steroids extracted from calf urine were calculated using calibration standards in the range of 5-200 ng (injected amount). From the chromatograms the average noise was determined for both calibration standards and urine samples. The injected amount calculated at three times the noise level for calibration standards was taken as the LOD of the HPLC-DAD system. The average blank signal of four calf urine samples plus three times the noise level for urine samples or the standard deviation of the four blank signals depending on the number of positive blank samples was used to calculate the LODs for steroids extracted from calf urine. The results are summarised in Table 7.

For all steroids the LODs for calibration standards of the HPLC-system were below 10 ng injected amount. For 10 of the compounds this was also the case after extraction from calf urine. One of the samples contained a large interference at the retention time of MT, whereas in the other samples no matrix peak was observed. The high detection limit for E1, T, EE2 and P were also the result of interference. Two peaks eluting around the retention time of Zer made detection of this growth promoter difficult, but its metabolite Tal could be determined at lower levels although here too interference caused some problems. The LODs of the oestrogens were higher, because of the higher noise level in samples at 192 nm.

Limitations of and Potentials of the Procedure

An LOD of 10 ng injected amount is equivalent to 20 ng/ml in urine samples. Therefore, all detection limits reported here are higher than the requirement of EU legislation (0.5 ng/ml) [41], although the LODs for the HPLC-DAD system were sufficiently low [29]. However, the method may be suitable for use during the fattening of calves, when concentrations of the substances in urine are higher. When an autosampler is used in combination with the HPLC system, the redissolution volume can be chosen somewhat smaller and the detection limits may be lower for most of the compounds. This will only be the case for the steroids, where no interference was observed. An additional IAC extraction may be included in the procedure and this could result in cleaner extracts. Due to time restraints this was not tried.

Table 7. Detection limits (ng injected) of the anabolic steroids and related substances in the HPLC-DAD system and for the anabolic steroids extracted from calf urine samples.

steroid	HPLC	urine
Tal*	2	20
Tb	4	10
Zer*	2	80
ßNT	1	2
NT*	2	2
E2	8	9
EE2	9	34
T	1	54
E1	7	19
Zeara	1	4
MT	2	39**
DE	3	9
tDES	0.5	2
HEX	0.5	0.6
ClTdiol	1	2
cDES	0.5	43
P	0.5	18
MP	1	2
MED	0.5	3

* no extensive experiments with urine samples could be performed due to time limitations and detection limits are estimated

** based on one sample with an interfering peak; other blanks were negative resulting in a detection limit of 3 ng

From Table 5 it becomes clear that there are no false positives for Tb in calf urine. For the urine samples of adult animals, no false positive results were obtained either. Also, only two cow urine samples gave false negatives at a spiking level just below the detection limit. The repeatability, reproducibility, accuracy and precision at a spiking level of 10 ng/ml and 25 ng/ml were good (Tables 3 and 4). Therefore, this method appears to be suitable for the quantitative analysis of Tb in all bovine urine samples. However, the detection limit, which is currently about 10-20 ng/ml depending on the noise level, is too high to meet the requirements in the EU legislation [41]. It may be

possible to increase the sample volume to solve this problem. It should be noted that in adult urine samples a peak was observed at the retention time of Tb at 230 nm, but this does not interfere with the analysis at 350 nm. Yet, when UV spectra are used for identification of the substance, Tb should be separated from this interference. This may be achieved by changing the percentage of acetonitrile in the mobile phase or possibly by performing a pre-extraction with Extrelut columns, which is already included in other procedures developed for the present project [42,43]. Several preliminary experiments with Extrelut showed adequate recoveries of Tb (over 80%) and somewhat cleaner extracts of cow urine could be obtained. The pre-extraction should be performed after the deconjugation step, because conjugates of DES could not be recovered from the column. Because of time limitations, this option was not investigated further.

One method has been reported for the extraction of several androgens from human urine using on-line extraction with a C2 column followed by LC-MS analysis. This method is claimed to be suitable for 28 anabolic steroids, including several metabolites. No LODs are given, but 100 ng steroid in 0.2 ml urine could be detected [15]. Another method was described for the analysis of three sulfoconjugated androgens in equine urine using C18 SPE with HPLC-UV detection [44]. Other reported SPE methods are single residue methods [18] or use GC-MS [16,19,20,24-26], GC-MS-MS [21], GC-FID [17] or immuno-assays [22,23,26] for the detection of the steroids. IAC uses the more specific interaction between analyte and antibody for the extraction of the substance of interest from the matrix. However, for multi-residue analysis either a good cross reactivity of the antibody with all analytes should exist or a combination of antibodies should be used. An example of the first strategy is the use of the salbutamol antibody in a multi-residue method for the screening of the beta-agonists. For the analysis of the anabolic steroids no single antibody is available that can extract all substances of interest. Therefore, at least six antibodies must be combined in a multi-IAC column and even then not all steroids are retained [28].

In conclusion, the SPE approach reported here can be used as a multi-residue method for the analysis of at least 19 illegally used anabolic steroids and related substances or their metabolites in calf urine. The detection limits of these steroids are still too high to meet the requirements of the EU legislation, but the method may be suitable for use during fattening, when higher concentrations of the substances are expected. The method is suitable for the quantitative determination of Tb in urine of calves and adult animals.

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